[Claim 6] The transformant of claim 1, wherein the NADPH-acetoacetyl CoA reductase gene comprises a DNA encoding the following protein (a) or (b):

(a) a protein having an amino acid sequence represented by SEQ ID NO:8, or

(b) a protein having an amino acid sequence including deletion, substitution or addition of one or more amino acids relative to the amino acid sequence represented by SEQ ID NO: 8, and having NADPH-acetoacetyl CoA reductase activity.

[Claim 7] The transformant of claim 1, wherein the NADPH-acetoacetyl CoA reductase gene comprises the following DNA (a) or (b):

(a) a DNA having a nucleotide sequence represented by SEQ ID NO: 7, or

(b) a DNA hybridizing to a DNA containing a nucleotide sequence of SEQ ID NO: 7 under stringent conditions, and encoding a protein with NADPH-acetoacetyl CoA reductase activity.

[Claim 8] The transformant of claim 1 which is a bacterium belonging to the genus *Pseudomonas* or the genus *Ralstonia*.

[Claim 9] The transformant of claim 8, wherein the bacterium belonging to the genus *Pseudomonas* is *Pseudomonas* sp. strain 61-3 (JCM10015).

[Claim 10] A method of producing copolymer polyester which comprises the steps of culturing the transformant of any one of claims 1 to 9, and collecting polyester from the culture product.

[Claim 11] The method of producing copolymer polyester of claim 10, wherein the polyester comprises 3-hydroxyalkanoic acid units with a carbon number of 4 to 12.

[Claim 12] The method of producing copolymer polyester of claim 11, wherein the 3-hydroxyalkanoic acid units contain 3-hydroxybutanoic acid with 80 to 95% molar fraction.

[Detailed Description of the Invention]

[0001]

[Field of the Invention]

The present invention relates to a transformant that is obtained by transforming a host, whose polyhydroxybutanoic acid polymerase gene is disrupted, with a recombinant vector containing a polyester polymerase gene, a β-ketothiolase gene, and a NADPH-acetoacetyl CoA

reductase gene, and a method of producing copolymer polyester using the transformant.

[0002]

[Prior Arts]

Many microorganisms are known to bio-synthesize poly-3-hydroxybutanoic acid (P(3HB)) and accumulate the fine granular products in its cells as energy storage materials. P(3HB) extracted from microorganisms is a thermoplastic polymer having a melting temperature around 180℃. Currently, P(3HB) is receiving attention as an eco-friendly plastic for environmental conservation because of its good biodegradation ability and biological compatibility. Moreover, P(3HB) can be synthesized from regenerative carbon sources including sugar and vegetable oil using various microorganisms. On the other hand, P(3HB) has a poor impact resistance since it is a high crystallinity polymer. This physical property has hindered the commercialization of P(3HB). However, the impact resistance of P(3HB) can be improved by producing a copolymer of long chain 3-hydroxyalkanoic acid (3HA) units and 3HB unit, so as to produce a flexible material. For example, random copolymer polyester P(3HB-co-3HV) of 3HB and 3-hydroxyvaleric acid (3HV) with a carbon number of 5, also known as Biopol™, is synthesized by cultivation the bacteria *Ralstonia eutropha* (previously known as *Alcaligenes eutrophus*) in medium supplemented with glucose as a carbon source and propionic acid (European Patent Application No. 0052459, 1981). In addition, a random copolymer P(3HB-co-3HH) of 3HB and 3-hydroxyhexanoic acid (3HH) is synthesized by *Aeromonas caviae*. This copolymer and its production method have been studied and developed as described in Japanese Patent Laid Open Publication Nos. 5-93049 and 7-265065. P(3HB-co-3HH) copolymer has been shown to be a flexible polymer material because its crystallinity decreases as 3HH unit composition increases. Furthermore, P(3HB-co-3HH) copolymer has a good thermostability and mold ability so that it can be processed into a strong string, or into a transparent, flexible film (Y. Doi, S. Kitamura, H. Abe, Macromolecules 28, 4822-4823, 1995).

[0003]

Pseudomonas sp. strain 61-3 (JCM 10015) is known to contain polyester polymerases including PhaC1 (Japanese Laid Open Publication No. 10-276781, J. Bacteriol., 180, 6459-6467,

1998) and PhaC2 (J. Bacteriol., 180, 6459-6467, 1998), which can use various 3HA units whose carbon number ranges from 4 to 12 as substrates. However, copolymer polyester produced by *Pseudomonas* sp. strain 61-3 is not a preferable plastic material because it becomes amorphous due to its low 3HB composition.

[0004]

[Objects to be Achieved by the Invention]

An object of the present invention is to provide a transformant that is obtained by transforming a host, whose polyhydroxybutanoic acid polymerase gene is disrupted, with a recombinant vector containing a polyester polymerase gene, a β-ketothiolase gene, and a NADPH-acetoacetyl CoA reductase gene, and a method of producing a copolymer polyester with a high composition of 3HB using the transformant.

[0005]

[Means to Achieve the Objects]

As a result of thorough studies on the above problems, the present inventors have completed the invention by finding that transformants obtained by transforming *Pseudomonas* sp. strain 61-3, whose polyhydroxybutanoic acid polymerase gene is disrupted, with a recombinant vector containing a polyester polymerase 1 gene derived from *Pseudomonas* sp. strain 61-3 (*phaC1* gene), a β-ketothiolase gene (*phbA* gene) derived from *Ralstonia eutropha*, and a NADPH-acetoacetyl CoA reductase gene (*phbB* gene) derived from *Ralstonia eutropha*, produce a P(3HB-co-3HA) having molar composition of 3HB ranges from 80 to 95% and having 3HA units with a carbon number of 4 to 12.

In other words, the present invention provides a transformant that is obtained by transforming a host, whose polyhydroxybutanoic acid polymerase gene is disrupted, with a recombinant vector containing a polyester polymerase gene, β-ketothiolase gene, and NADPH-acetoacetyl CoA reductase gene.

[0006]

The present invention provides a transformant wherein the polyester polymerase gene comprises a DNA encoding the following protein (a) or (b):

(a) a protein having an amino acid sequence represented by SEQ ID NO: 2 or 4, or

(b) a protein having an amino acid sequence including deletion, substitution, or addition of one or more amino acids relative to the amino acid sequence represented by SEQ ID NO: 2 or 4, and having polyester polymerase activity.

[0007]

Further, the present invention provides a transformant wherein the polyester polymerase gene comprises the following DNA (a) or (b):

(a) a DNA containing a nucleotide sequence represented by SEQ ID NO: 1 or 3, or

(b) a DNA hybridizing to a DNA containing a nucleotide sequence of SEQ ID NO: 1 or 3 under stringent conditions, and encoding a protein with polyester polymerase activity.

[0008]

Further, the present invention provides a transformant wherein the β-ketothiolase gene comprises a DNA encoding the following protein (a) or (b):

(a) a protein having an amino acid sequence represented by SEQ ID NO: 6, or

(b) a protein having an amino acid sequence including deletion, substitution, or addition of one or more amino acids relative to the amino acid sequence represented by SEQ ID NO:6, and having β-ketothiolase gene activity.

[0009]

Further, the present invention provides a transformant wherein the β-ketothiolase gene comprises the following DNA (a) or (b):

(a) a DNA having an nucleotide sequence represented by SEQ ID NO: 5, or

(b) a DNA hybridizing to a DNA containing a nucleotide sequence of SEQ ID NO: 5 under stringent conditions and encoding a protein with β-ketothiolase activity.

[0010]

Further, the present invention provides a transformant wherein the NADPH-acetoacetyl CoA reductase gene comprises a DNA encoding the following protein (a) or (b):

(a) a protein having an amino acid sequence represented by SEQ ID NO:8, or

(b) a protein having an amino acid sequence including deletion, substitution or addition of one or more amino acids relative to the amino acid sequence represented by SEQ ID NO: 8, and

having NADPH-acetoacetyl CoA reductase activity.

[0011]

Furthermore the present invention provides a transformant wherein the NADPH-acetoacetyl CoA reductase gene comprises the following DNA (a) or (b):

(a) a DNA having a nucleotide sequence represented by SEQ ID NO: 7, or

(b) a DNA hybridizing to a DNA containing a nucleotide sequence of SEQ ID NO: 7 under stringent conditions, and encoding a protein with NADPH-acetoacetyl CoA reductase activity.

[0012]

Furthermore, the present invention provides a transformant which is a bacterium belonging to the genus *Pseudomonas* or *Ralstonia*. An example of the bacterium belonging to the genus *Pseudomonas* is the genus *Pseudomonas* sp. strain 61-3.

The present invention also provides a method of producing copolymer polyester which comprises the steps of culturing a transformant, and collecting polyester (for example, a polyester comprising 3-hydroxyalkanoic acid units with a carbon number of 4 to 12) from the culture product. An example of the polyester produced herein comprises 3-hydroxyalkanoic acid units with a carbon number of 4 to 12 wherein molar composition of 3-hydroxybutanoic acid is 80 to 95% molar fraction.

Detailed description of this invention will now be given as follows.

[0013]

[Mode of Carrying Out the Invention]

1. A Host for Transformation

Hosts that can be used for transformation are not specifically limited so far as they can express each gene contained in a recombinant vector. Examples of the host include bacteria belonging to the genus *Pseudomonas*, such as *Pseudomonas putida*, *Pseudomonas* sp. strain 61-3, those belonging to the genus *Ralstonia*, such as *Ralstonia eutropha*, those belonging to the genus *Bacillus*, such as *Bacillus subtilis*, those belonging to the genus *Escherichia*, such as *Escherichia coli*, yeast belonging to the genus *Saccharomyces*, such as *Saccharomyces cerevisiae*, yeast belonging to the genus *Candida*, such as *Candida maltosa*, and animal cells, such as COS cells and CHO cells.

[0014]

In particular, a cell whose certain naturally occuring polyester polymerase gene is disrupted can be used to allow the host cell to produce a polyester having a desired composition. Figure 1 shows the synthetic pathway of polyhydroxybutanoic acid (P(3HB)), and of a copolymer (P(3HB-co-3HA)) of 3-hydroxybutanoic acid and 3-hydroxyalkanoic acid. The *Pseudomonas* sp. strain 61-3, whose polyhydroxybutanoic acid polymerase gene is disrupted, can be constructed by deleting the terminus of a polyhydroxybutanoic acid polymerase gene to cause mutation in the gene, and introducing the mutated gene into *Pseudomonas* sp. strain 61-3 to cause homologous recombination between the mutated polyhydroxybutanoic acid polymerase gene and a polyhydroxybutanoic acid polymerase gene on a chromosome. Whether the polyhydroxybutanoic acid polymerase gene is disrupted or not can be confirmed by Southern hybridization using a part of the gene as a probe. That is, it can be confirmed by examining if a band of hybridization shifts to an expected position relative to a band of a wild type.

[0015]

2. Recombinant Vector

Recombinant vectors of this invention can be obtained by ligating (inserting) a polyester polymerase gene, β-ketothiolase gene, and NADPH-acetoacetyl CoA reductase gene into an appropriate expression vector.

Examples of the polyester polymerase gene include a *phaC1* gene or a *phaC2* gene derived from *Pseudomonas* sp. strain 61-3. A nucleotide sequence of the *phaC1* gene is shown in SEQ ID NO: 1, and an amino acid sequence of the polyester polymerase encoded by the *phaC1* gene is shown in SEQ ID NO: 2. Mutations including deletion, substitution, and addition of one or more amino acids may occur in the amino acid sequence so far as proteins having these amino acid sequences have polyester polymerase activity. For example, one, preferably 2 to 5, more preferably 5 to 10 amino acids may be deleted from the amino acid sequence represented by SEQ ID NO: 2 or 4; or one, preferably 2 to 5, more preferably 5 to 10 amino acids may be added to the amino acid sequence represented by 2 or 4; or one, preferably 2 to 5, more preferably 5 to 10 amino acids may be substituted in the amino acid sequence

represented by SEQ ID NO: 2 or 4. Alternatively, a DNA which can hybridize to a DNA having a nucleotide sequence represented by SEQ ID NO: 1 or 3 under stringent conditions can also be used in this invention so far as the DNA encodes a protein having polyester polymerase activity. The stringent conditions include a temperature of 60 to 68℃, preferably 55 to 68℃, and a sodium concentration of 250 to 350 mM, preferably 300 to 400 mM.

[0016]

An example of a β-ketothiolase gene is a *phbA* gene derived from *Ralstonia eutropha*. A nucleotide sequence of the *phbA* gene is shown in SEQ ID NO:5, and an amino acid sequence of the β-ketothiolase encoded by the *phbA* gene is shown in SEQ ID NO:6. So far as proteins having these amino acid sequences possess β-ketothiolase activity, mutations including deletion, substitution, and addition of one or more amino acids may occur in these amino acid sequences. For example, one, preferably 2 to 5, more preferably 5 to 10 amino acids may be deleted from the amino acid sequence represented by SEQ ID NO: 6; or one, preferably 2 to 5, more preferably 5 to 10 amino acids may be added to the amino acid sequence represented by SEQ ID NO: 6; or one, preferably 2 to 5, more preferably 5 to 10 amino acids may be substituted in the amino acid sequence represented by SEQ ID NO: 6. Alternatively, a DNA which can hybridize to a DNA having a nucleotide sequence represented by SEQ ID NO: 5 under stringent conditions can also be used in this invention so far as the DNA encodes a protein having β-ketothioesterase activity. The stringent conditions include a temperature of 60 to 68℃, preferably 55 to 68℃, and sodium concentration of 250 to 350 mM, preferably 300 to 400 mM.

[0017]

An example of a NADPH-acetoacetyl CoA reductase gene is a *phbB* gene derived from *Ralstonia eutropha*. A nucleotide sequence of the *phbB* gene is shown in SEQ ID NO:7, and an amino acid sequence of the NADPH-acetoacetyl CoA reductase encoded by the *phbB* gene is shown in SEQ ID NO: 8. So far as proteins having these amino acid sequences possess NADPH-acetoacetyl CoA reductase activity, mutations including deletion, substitution, and addition of one or more amino acids may occur in these amino acid sequences. For example, one, preferably 2 to 5, more preferably 5 to 10 amino acids may be deleted from the amino acid sequence represented by SEQ ID NO: 8; or one, preferably 2 to 5, more preferably 5 to 10

amino acids may be added to the amino acid sequence represented by SEQ ID NO: 8; or one, preferably 2 to 5, more preferably 5 to 10 amino acids may be substituted in the amino acid sequence represented by SEQ ID NO: 8. Alternatively, a DNA which can hybridize to a DNA having a nucleotide sequence represented by SEQ ID NO: 7 under stringent conditions can also be used in this invention so far as the DNA encodes a protein having NADPH-acetoacetyl CoA reductase activity. The stringent conditions include a temperature of 60 to 68℃, preferably 55 to 68℃, and sodium concentration of 250 to 350 mM, preferably 300 to 400 mM.

[0018]

Vectors used herein to insert each gene as described above are not specifically limited so far as they can autonomously replicate in hosts. Examples of the vector include plasmid DNA and phage DNA.

When *Escherichia coli* is used as a host cell, examples of vectors include plasmid DNAs, such as pBR322, pUC18, and pBluescript II, phage DNAs, such as EMBL3, M13, and λgt11. When yeast is used as a host cell, examples of vectors include YEp13 and YCp50; when an animal cell is used as a host cell, examples of vectors include pcDNAI, and pcDNAI/Amp (Invitrogen).

[0019]

In addition, when bacteria belonging to the genus *Ralstonia* and those belonging to the genus *Pseudomonas* are used as host cells, examples of vectors include pLA2917 (ATCC37355) having RK2 replication origin, and pJRD215 (ATCC 37533) having RSF1010 replication origin. These replication origins are replicated and retained in a broad range of hosts.

A gene can be inserted into a vector by integrating a DNA fragment with the above gene into a vector DNA fragment digested with a restriction enzyme. At this time, the above gene must be inserted into a vector so that the gene function is exhibited. Particularly, gene expression requires insertion of a gene downstream of a promoter. Any promoter can be used so far as it can express in a host. When *Escherichia coli* is used as a host cell, examples of promoters include trp promoter, lac promoter, PL promoter, PR promoter, and T7 promoter; when yeast is used as a host cell, examples of promoters include gal1 promoter and gal 10

promoter. When bacteria belonging to the genus *Pseudomonas* are used as host cells, a promoter region of upstream of a $phaC1_{Ps}$ gene or a $phbCAB_{Re}$ gene may be used as a promoter. A nucleotide sequence of upstream of the $phaC1_{Ps}$ gene is as shown in SEQ ID NO: 9, and that of upstream of the $phbCAB_{Re}$ operon is as shown in SEQ ID NO: 10.

[0020]

If necessary, a terminator, enhancer, splicing signal, polyA additional signal, selection marker, and ribosome binding sequence (SD) and the like may be integrated into the vector of this invention. Examples of the selection marker include ampicillin-, tetracycline-, neomycin-, kanamycin, and chroramphenicol-resistant genes. Particularly when bacteria belonging to the genus *Pseudomonas* are used as host cells, a terminator region of downstream of a $phbCAB_{Re}$ operon may be used as a terminator. A nucleotide sequence downstream of the $phbCAB_{Re}$ operon is as shown in SEQ ID NO: 11.

[0021]

3. Preparation of Transformants

The transformant of this invention can be obtained by introducing a recombinant vector obtained in 2 above into a host cell of 1 above. Examples of a method of introducing a recombinant DNA into a bacterium include a method using calcium ions (Current Protocols in Molecular Biology, vol. 1, p.1.8.1, 1994) or electroporation (Current Protocols in Molecular Biology, vol. 1, p.1.8.4, 1994). A plasmid can be introduced into a bacterium belonging to the genus *Pseudomonas* by the conjugation transfer method (Friedrich et al. : J. Bacteriol. 147: 198-205, 1981).

[0022]

Examples of a method of introducing a recombinant DNA into yeast include electroporation (Methods. Enzymol., 194:182-187, 1990), the spheroplast method (Proc. Natl. Acad. Sci., USA, 84:1929-1933 (1978)), the lithium acetate method (J. Bacteriol., 153:163-168 , 1983). Examples of a method of introducing a recombinant DNA into an animal cell include electroporation and the calcium phosphate method.

[0023]

The transformant (strain) *Pseudomonas* sp. BB49 obtained by transforming *Pseudomonas* sp. strain 61-3, whose polyhydroxybutanoic acid polymerase gene is disrupted, with a plasmid pJBB49-*phb*, and the transformant *Pseudomonas* sp. KSc46 obtained by transforming with a plasmid pJKSc46-*pha*, and the transformant *Pseudomonas* sp. KSc54 obtained by transforming with a plasmid pJKSc54-*phb* were deposited with National Institute of Bioscience and Human-Technology, National Institute of Advanced Industrial Science and Technology (1-1-3, Higashi 1-chome, Tsukuba-shi, Ibaraki-ken, Japan) on August 5, 1999. The accession numbers received were FERM P-17503, FERM P-17504 and FERM P-17505, respectively.

[0024]

4. Production of Polyester

Polyester is produced by culturing the transformants of this invention in media, allowing them to synthesize and accumulate copolymer polyester in the culture cells or culture products, and collecting the polyester from the culture cells or culture products.

The transformants of this invention are cultured in media by standard methods employed for culturing hosts.

[0025]

Examples of culture media for culturing transformants obtained using bacteria belonging to the genus *Ralstonia* or the genus *Pseudomonas* as host cells include media containing carbon sources assimilable by microorganisms, and containing a limited amount of any one of nitrogen sources, inorganic salts, or other organic nutrient sources. For example, a medium used herein contains nitrogen sources limited to 0.01% to 0.1%. Culture is performed at 25 to 37℃ aerobically for 2 to 7 days to allow the cells to accumulate polyester within the cells, followed by collection of the polyester.

[0026]

Examples of carbon sources include carbohydrates such as glucose, fructose, sucrose, and maltose. Oil and fat-related substances with a carbon number of 4 or more than 4 may be used as carbon sources. Examples of such carbon sources include natural oil, such as corn oil, soy oil, safflower oil, sunflower oil, olive oil, palm oil, colza oil, fish oil, whale oil, pig oil, or

cattle oil; fatty acids, such as butanoic acid, pentanoic acid, hexanoic acid, octanoic acid, decanoic acid, lauric acid, oleic acid, palmitin acid, linoleic acid, linolic acid or myristic acid, or esters of these fatty acids; octanol, lauryl alcohol, oleyl alcohol, or palmityl alcohol, or esters of these alcohols.

[0027]

Examples of nitrogen sources include ammonia, ammonium salts such as ammonium chloride, ammonium sulfate, and ammonium phosphate in addition to peptone, meat extract, yeast extract, corn steep liquor. Examples of inorganic substances include potassium phosphate, potassium secondary phosphate, magnesium phosphate, magnesium sulfate, sodium chloride.

[0028]

Normally, shake-culture is performed under aerobic conditions at 25 to 37℃ for 24 hours or more following induction of expression. Antibiotics including kanamycin, ampicillin, and tetracycline may be added to media while culturing.

When a transformant (microorganisms) transformed by an expression vector containing an inducible promoter is cultured, an inducer may be added to the medium. For example, isopropyl-β-D-thiogalactoside (IPTG), indoleacrylic acid (IAA) and the like may be added to the media.

Examples of media for culturing transformants obtained using animal cells as host cells include RPMI-1640 media, DMEM media, or those supplemented with fetal calf serum. Culturing is usually performed in the presence of 5% CO_2 at 30 to 37℃ for 14 to 28 days. Antibiotics including kanamycin and penicillin may be added to the media.

[0029]

Polyester of this invention can be purified as follows. Transformants are collected by centrifugation from culture solution, washed with distilled water, and then dried. Next, the dried transformants are suspended in chloroform and heated to extract polyester. Residue is removed by filtration. Then methanol is added to the chloroform solution to precipitate polyester. Following removal of supernatant by filtration and centrifugation, the product is

dried to obtain purified polyester. The resulting polyester can be used as materials for biodegradable strings, films, and various containers. Whether the resulting polyester is of interest or not is confirmed by standard techniques including gas chromatography and nuclear magnetic resonance methods.

[0030]

[Examples]

A detailed description of the present invention will be given as follows. However, the technical scope of the present invention is not limited by the examples.

Example 1 Establishment of a polyhydroxybutanoic acid polymerase gene-disruptant of *Pseudomonas* sp. strain 61-3

Pseudomonas sp. strain 61-3 (JCM 10015) contains polyhydroxybutanoic acid polymerase (PhbC) in addition to polyhydroxyalkanoic acid polymerase (PhaCl and PhaC2) that can use a broad range of substrates with a carbon number of 4 to 12. Therefore, this strain 61-3 synthesizes a blend of polyester (P(3HB)) having a sole unit of 3-hydroxybutanoic acid and copolymer polyester (P(3HB-co-3HA)) containing 3HA units with a carbon number of 4 to 12. A 3HB unit is often favored in comparison to PhaC1 or PhaC2 as a substrate by PhbC, which has a high affinity with the 3HB unit, so that copolymer polyester with a high 3HB molar composition is not synthesized. Accordingly, a polyhydroxybutanoic acid polymerase gene-disruptant of *Pseudomonas* sp. strain 61-3 was established.

[0031]

First, the 5' terminal region of 342 bp and the 3' terminal region of 418 bp were deleted from a gene (*phbC*$_{Ps}$ gene) encoding polyhydroxybutanoic acid polymerase of *Pseudomonas* sp. strain 61-3, thereby preparing a deleted polyhydroxybutanoic acid polymerase gene fragment (*Eco*RI-*Pst*I fragment) of 941 bp. Next, the resulting *Eco*RI-*Pst*I fragment was ligated to *Eco*RI and *Pst*I sites of pBR322, thereby constructing a plasmid pBREP9 (Tc^r) for disrupting polyhydroxybutanoic acid polymerase gene. The obtained plasmid pBREP9 (Tc^r) was introduced into *Pseudomonas* sp. strain 61-3 suspended in 8 mM HEPES buffer (pH 7.2) containing 272 mM sucrose by electroporation (conditions: 7.5 kV/cm, 800 Ω, 25 μF). *phbC*$_{Ps}$ gene-disrupted strain (*phbC*::*tet*) was screened for strains capable of growing on LB

media containing tetracyclin. Then chromosomal DNAs prepared from some of the screened strains and wild strains were digested with appropriate restriction enzymes. Following digestion, Southern hybridization with a part of $phbC_{Ps}$ gene as a probe was performed, thereby selecting and obtaining strains showing a band that had shifted to a position of an expected molecular weight.

[0032]

Example 2 Construction of a Recombinant Vector

Figure 2 shows steps to construct a recombinant vector. Cleavage and ligation of DNA fragments were performed according to standard techniques (Sambrook et al. : Molecular Cloning, A Laboratory Manual 2nd ed., Cold Spring Harbor Laboratory Press, 1989). As shown in Fig. 2, pBSEX22 and pGEM'-phbCAB were used as start plasmids. Here, pBSEX22 was constructed by inserting a 2.2-kb *Eco*RI-*Xba*I region containing *phaC1* gene of *Pseudomonas* sp. strain 61-3 into pBluescript II KS+. pGEM'-phbCAB was constructed by amplifying *phbCAB* gene of *Ralstonia eutropha* H16 (ATCC 17699) (J. Biol. Chem., 264, 15293-15297, 1989; J. Biol. Chem., 264, 15298-15303, 1989) by PCR and inserting the resulting PCR fragment into a pGEM-T (Promega) vector having disrupted *Nde*I and *Pst*I sites.

[0033]

At the final step of the construction of a recombinant vector, the DNA fragment encoding a gene of interest was inserted and ligated into a plasmid pJRD215 (ATCC37533) capable of replicating within *Pseudomonas* sp. strain 61-3. In other words, a plasmid pJASc22 was constructed by cutting out a *Apa*I-*Sac*I fragment which contains polyester polymerase 1 gene ($phaC1_{Ps}$ gene) derived from *Pseudomonas* sp. strain 61-3 and a promoter (P_{Ps} promoter) of this gene from pBSEX22, and by inserting and ligating the fragment into *Apa*I and *Sac*I sites of pJRD215. Further, a plasmid pJBB49-*phb* was constructed by inserting and ligating *Bam*HI-*Bam*HI fragment which contains $phbCAB_{Re}$ operon promoter (P_{Re} promoter) derived from *Ralstonia eutropha*, $phaC1_{Ps}$ gene, β-ketothiolase gene ($phbA_{Re}$ gene) derived from *Ralstonia eutropha*, NADPH-acetoacetyl CoA reductase gene ($phbB_{Re}$ gene) derived from *Ralstonia eutropha*, and $phbCAB_{Re}$ operon terminator (T_{Re} terminator) derived from *Ralstonia eutropha* into a *Bam*HI site of pJRD215. Furthermore, a plasmid pJKSc46-*pha* was constructed

by inserting and ligating *Kpn*I-*Sac*I fragment, which contains P_{Ps} promoter, *phaC1*$_{Ps}$ gene, *phbA*$_{Re}$ gene, *phbB*$_{Re}$ gene and T_{Re} terminator into *Kpn*I and *Sac*I sites of pJRD215. Moreover, a plasmid pJKSc54-*phab* was constructed by inserting and ligating *Kpn*I-*Sac*I fragment, which contains P_{Ps} promoter, *phaC1*$_{Ps}$ gene, P_{Re} promoter, *phbA*$_{Re}$ gene, *phbB*$_{Re}$ gene and T_{Re} terminator, into *Kpn*I and *Sac*I sites of pJRD215. Structures of the four types of plasmids obtained are shown in Fig. 3.

[0034]

Example 3 Construction of a *Pseudomonas* sp. strain 61-3 (*phbC*::*tet*) Transformant

A transformant was constructed by introducing the plasmid obtained in Example 2 into the *phbC*$_{Ps}$ gene-disruptant (*phbC*::*tet*) of *Pseudomonas* sp. strain 61-3 obtained in Example 1 by the conjugation transfer method. That is, four types of plasmids pJASc22, pJBB49-*phb*, pJKSc46-*pha*, and pJKSc54-*phab* were separately transformed into *E.coli* strain S17-1 by the calcium chloride method. Next, the obtained transformants and *Pseudomonas* sp. strain 61-3 (*phbC*::*tet*) were separately cultured overnight in 1.5 ml of LB medium at 37℃ and 28℃, respectively. Subsequently, 0.1 ml of the culture product of *E.coli* and 0.1 ml of that of *Pseudomonas* sp. strain 61-3 (*phbC*::*tet*) were mixed and cultured for 4 hours at 28℃. After culturing, the mixture was plated over MS agar media (0.9% sodium diphosphate, 0.15% monopotassium phosphate, 0.05% ammonium chloride, 2% glucose, 0.1% (v/v) Trace element solution ($CoCl_2 \cdot 6H_2O$ 0.218g, $FeCl_3$ 9.7g, $CaCl_2$ 7.8g, $NiCl_3 \cdot 6H_2O$ 0.118g, $CrCl_3 \cdot 6H_2O$ 0.105g, and $CuSO_4 \cdot 5H_2O$ 0.156g dissolved in 1 liter of 0.1N hydrochloric acid), 1.5% agar, 50mg/l kanamycin, 12.5 g/l tetracyclin) and cultured for 2 to 5 days at 28℃.

Transformants were obtained by isolating colonies that had grown on the MS agar medium. Four types of transformants having pJASc22, pJBB49-*phb*, pJKSc46-*pha*, and pJKSc54-*phab* were designated as *Pseudomonas* sp. strain ASc22, BB49, KSc46, and Ksc54, respectively.

[0035]

Example 4 Polyester Synthesis by *Pseudomonas* sp. strain 61-3 (*phbC*::*tet*) Transformants

Polyester was produced by transformants obtained in Example 3. First, each strain of *Pseudomonas* sp. 61-3 (*phbC*::*tet*), ASc22, BB49, KSc46, and KSc54 was inoculated in 100 ml

of MS medium containing 2% glucose, and then cultured in Sakaguchi flasks (shaking flasks) for 48 hours at 28℃. Cells were collected by centrifugation, washed with distilled water, and freeze-dried. The dried cells were measured for weight, polyester content, and polyester composition.

[0036]

That is, 2 ml of a mixture of sulfuric acid – methanol (15:85) and 2 ml of chloroform were added to 10 to 30 mg of the dried cells, then the containers were tightly stoppered. Then, methyl ester was obtained by decomposing intracellular polyester by heating for 140 minutes at 100℃. Then 1ml of distilled water was added to the methyl ester, followed by vigorous stirring. Next the mixture was allowed to stand to separate into two layers. The organic layer of the lower layer was taken out and the composition was analyzed by capillary gas chromatography. A gas chromatograph used herein was GC-17A (manufactured by SHIMADZU CORPORATION) and capillary column was NEUTRA BOND-1 (manufactured by GL Science, column length 25 m, column internal diameter 0.25 mm, liquid film thickness 0.4 μm). Temperature was raised at a rate of 8℃/min from the initial temperature of 100℃. Table 1 shows the results.

[0037]

[Table 1]

Copolymer polyester production by *Pseudomonas* sp. strain 61-3 (*phbC::tet*)

Strain	Dry cell weight (g/l)	Polyester content (wt %)	Polyester composition (mol%)					
			3HB (C_4)	3HHx (C_6)	3HO (C_8)	3HD (C_{10})	3HDD (C_{12})	3H5DD (C_{12})
phbC::tet	0.7	4	36	0	6	23	20	15
Asc22	0.7	6	64	0	2	15	11	8
BB49	1.7	38	92	0	1	4	2	1
Ksc46	2.6	37	81	0	1	9	5	4
Ksc54	2.5	45	92	0	1	3	3	1

Note) 3HB: 3-hydroxybutanoic acid; 3HHx: 3-hydroxyhexanoic acid; 3HO: 3-hydroxyoctanoic acid, 3HD: 3-hydroxydecanoic acid; 3HDD: 3-hydroxydodecanoic acid, 3H5DD: 3-hydroxy-cis-5-dodecanoic acid